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Synaptic Integrins in Developing, Adult, and Mutant Muscle: Selective Association of α1, α7A, and α7B Integrins with the Neuromuscular Junction

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Differentiation of both pre- and postsynaptic structures at the skeletal neuromuscular junction is organized by the basal lamina that occupies the synaptic cleft. As β1 integrins are a major class of receptors for basal lamina components, we stained muscles with antibodies to the 10 integrin α subunits known to form dimers with β1, to determine if any of these molecules were concentrated at synaptic sites on muscle fibers. In both developing and adult muscle, the integrin α1 chain was selectively associated with presynaptic cells (Schwann cells and/or nerve terminals), while α7 was present on both synaptic and extrasynaptic portions of the muscle fiber surface. Thus α1 and α7 integrins are present in synaptic membranes. Expression of the α7 chain was analyzed further by staining with antibodies specific for three alternatively spliced products of the α7 gene (A, B, and C), all of which were expressed in muscle. The α7A and α7B isoforms were confined to synaptic sites in adult muscle, while α7C was present both synaptically and extrasynaptically. In developing muscle, α7A appeared postnatally and specifically at the synapse; α7B was present throughout the muscle fiber perinatally, becoming confined to the synapse in the second postnatal week; and α7C was present extrasynaptically both perinatally and in adulthood. Thus, two of the α7 integrins are synapse-specific, and all three show distinct spatiotemporal patterns of expression within a single cell type. Finally, we asked whether perturbation of laminin expression affected the distribution of the α7 integrins. In normal mice, laminin β2 is concentrated in synaptic basal lamina. In β2-null mutant mice, α7A was still present at synaptic sites, but α7B was absent. This result provides genetic evidence that basal lamina composition is a determinant of integrin distribution.

INTRODUCTION

Formation of the neuromuscular junction involves a series of interactions between the pre- and postsynaptic cells (Hall and Sanes, 1993). Some of the signals that mediate this interchange have now been identified: they include the glycoproteins agrin and ARIA (heregulin/neuregulin), which are synthesized by motor neurons and induce differentiation of the postsynaptic apparatus (Falls *et al.*, 1993; Bowe and Fallon, 1995), and laminin β2 (s-laminin), which is synthesized by the muscle fiber and regulates differentiation of the nerve terminal (Noakes *et al.*, 1995a). Extracellular forms of all three of these molecules are stably bound in the basal lamina that occupies the synaptic cleft between the nerve terminal and the muscle fiber (Sanes, 1995). Other synaptic specializations of the basal lamina, for which functions have yet to be identified, include a laminin α1-like chain and the α3–5 chains of collagen IV. Extrasynaptic basal lamina, by contrast, is nearly devoid of agrin, ARIA, laminins α1 and β2, and collagens α3–5(IV); instead, it con-

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tains laminins α2 and β1 and collagens α1,2(IV) (Sanes *et al.,* 1990; Miner and Sanes, 1994; Chu *et al.,* 1995; Jo *et al.,* 1995).

To understand how synaptic basal lamina forms and functions, it is crucial to identify the cellular receptors with which its components interact. Candidate receptors for agrin (Bowe and Fallon, 1995) and ARIA (Jo *et al.,* 1995; Moscoso *et al.,* 1995b) have been identified on the muscle membrane, but none are known for the synaptic isoforms of laminin or collagen IV. Obvious candidates in this regard are the integrins, a family of heterodimeric ($\alpha\beta$) transmembrane proteins that serve as signal transducing receptors for a wide variety of extracellular matrix proteins, including laminins and collagens (Hynes, 1992; Sonnenberg, 1993). Integrins also bind cytoskeletal proteins through their cytoplasmic domains (Clark and Brugge, 1995) and could therefore serve to link the specialized cytoskeleton that underlies the postsynaptic membrane at the neuromuscular junction (Hall and Sanes, 1993) to the specialized extracellular matrix that overlies it.

The aim of the present study was to identify integrins concentrated at the neuromuscular junction. Our starting point was the observation that integrin β1, the only β subunit identified to date in muscle cells, is present in both synaptic and extrasynaptic portions of the muscle fiber membrane (Bozyczko *et al.,* 1989). β1 has been shown to form dimers with 10 different α subunits, α1–9 and αv. Eight of these, α1,3–7,9, and v have been reported to be present in developing skeletal muscle (Hirsch *et al.,* 1994; Terpe *et al.,* 1994; Blaschuk and Holland, 1994; Enomoto *et al.,* 1993; Lakonishok *et al.,* 1992; Palmer *et al.,* 1993; George-Weinstein *et al.,* 1993; Bao *et al.,* 1993; Song *et al.,* 1992; Rosen *et al.,* 1992; Duband *et al.,* 1992; Bronner-Fraser *et al.,* 1992; Steffenen *et al.,* 1992; McDonald *et al.,* 1995) but few have been sought at the neuromuscular junction, and none have been reported to be present at that site. Accordingly, we stained sections of adult muscle with a panel of antibodies specific for each of the 10 β1-associated α integrin subunits. Of these we found that two, α1 and α7, were concentrated at synaptic sites. Further analysis revealed that α1 was associated with presynaptic structures and α7 with the postsynaptic apparatus. Next, we generated antisera specific for each of three α7 isoforms that have recently been shown to be encoded by alternatively spliced products of the α7 gene (Collo *et al.,* 1993; Song *et al.,* 1993; Ziober *et al.,* 1993). Using these antibodies, we found that all three isoforms are present in adult muscle, but that each has a unique distribution and developmental pattern. Finally, we assessed the distribution of α7 in mutant mice that lack laminin β2 (Noakes *et al.,* 1995a) and found that the isoforms are differentially affected in the mutants. Together, these results demonstrate the existence of synaptic integrins, provide candidate receptors for synaptic basal lamina components, and demonstrate differential regulation of three products of a single integrin gene within a single cell type.

MATERIALS AND METHODS

Antibodies

Four mouse monoclonal antibodies to human integrins were obtained from Telios Pharmaceuticals (San Diego, CA): anti-α2 (clone P1E6; Wayner *et al.,* 1988), anti-α3 (clone P1B5; Wayner and Carter, 1987), anti-α4 (clone P4G9; Wayner *et al.,* 1989), and anti-α5 (clone P1D6; Wayner *et al.,* 1988). Rat anti-mouse α6 (clone GoH3; Sonnenberg *et al.,* 1987), which cross-reacts with human α6, was obtained from Coulter (Miami, FL). Mouse anti-human αv (clone VNR147; Freed *et al.,* 1989) was obtained from GIBCO-BRL (Gaithersburg, MD). Mouse anti-human β1 (clone 4B4; Morimoto *et al.,* 1985) was obtained from AMAC (Westbrook, ME). Mouse anti-human α1 (clone TS2/7; Hemler *et al.,* 1984) was a gift of Martin Hemler (Dana Farber Cancer Inst., Boston, MA). Mouse anti-rat α1 (clone 3A3; Tawil *et al.,* 1990) was a gift from Sal Carbonetto (McGill University, Montreal). Rabbit anti-human α8 (Schnapp *et al.,* 1995) and α9 (Palmer *et al.,* 1993) were gifts of Lynn Schnapp and Dean Sheppard (University of California, San Francisco), respectively. Mouse anti-rat α7, clones H36, and O26 were described by Song *et al.* (1992).

To generate antibodies specific for individual α7 isoforms, eight peptide sequences were deduced from α7A–C cDNAs (Song *et al.,* 1993; Ziober *et al.,* 1993). Two peptides were specific for α7A sequence, five for α7B, and one for α7C. Sequences used were: NH_2-CGWDSSSGRSTPRPPCPSTTQ (peptide 7A from α7A), NH_2-NSPSSSFRTN (peptide 22774 from α7A), NH_2-CEDRQQFKEEKTGTIQRSNWGNSQWEG (peptide 7B1 from α7B), NH_2-CGSDAHPILAADWHPELGPDGHPVSVTA (peptide 7B2 from α7B), (NH_2-QYHAVKIP REDRQQFKEEK (peptide 1214 from α7B), NH_2-GTIQRSNWGNSQWEGSDAH (peptide 1211 from α7B), NH_2-PELGPDGHPVPATA (peptide 914 from α7B) and NH_2-CAVPAQRILSIY (peptide 7C from α7C). The peptides were synthesized using t-BOC chemistry, purified, and coupled to keyhole limpet hemocyanin using maleimide (7A, 7B1, 7B2, and 7C) or carbodiimide (914, 1211, 1214, and 22774). Antisera were produced from these hemocyanin peptide conjugates by intramuscular and subcutaneous immunization of New Zealand White rabbits. Serum titers were monitored by ELISA, immunoblotting, and immunoprecipitation. In some cases, antisera were depleted of lipid with 0.25% sodium dextran sulfate and 1% $CaCl_2$, centrifuged at 10,000*g* for 10 min, precipitated with 50% ammonium sulfate, and dialyzed extensively against phosphate-buffered saline (PBS). All of these antisera except anti-22774, which reacted with authentic α7A on immunoblots, but failed to stain tissue, were used in this study.

Antibodies to laminin-1 and laminin β2 were generated in our laboratory and have been described previously (Sanes *et al.,* 1990). Rat monoclonal antibody 5A2 to mouse laminin-1 was obtained from Dale Abrahamson (University of Alabama; Abrahamson *et al.,* 1989) and found to be specific for laminin β1 by use of recombinant proteins (Martin *et al.,* 1995). A monoclonal antibody to SV2 was kindly provided by Kathy Buckley (Harvard University, Boston, MA; Feaney *et al.,* 1992). Fluorescein- and rhodamine-conjugated second antibodies were obtained from Boehringer-Mannheim Biochemicals (Indianapolis, IN) or Cappel/Organon Teknica (Durham, NC). Rhodamine-conjugated α-bungarotoxin was purchased from Molecular Probes (Eugene, OR).

Tissues

Diagnostic biopsies of human muscle were provided by Dr. Alan Pestronk (Department of Neurology, Washington University Medi-

cal Center). Biopsies were obtained for evaluation of muscle weakness. Three that revealed no histological or histochemical abnormalities upon examination by neurologists were used as normal muscle. All of our observations were reproduced on material from all three individuals. Pregnant rats were obtained from Harlan Bioproducts (Indianapolis, IN). To denervate muscles, rats were anesthetized and the sciatic nerve was cut in mid-thigh. Incisions were closed with sutures or surgical staples. Five or 7 days after surgery, animals were euthanized and muscles harvested. Mice in which the laminin β2 gene had been disrupted by homologous recombination have been described previously (Noakes *et al.*, 1995a,b).

Immunohistochemistry

Muscles were frozen in liquid nitrogen-cooled isopentane and cross-sectioned at 4–8 μm in a cryostat. Sections were incubated for 1 hr at room temperature with PBS supplemented with 10% normal goat serum. Sections were then incubated in the above buffer with primary antibody for 1–2 hr at room temperature, washed in PBS, reincubated with fluorescein-conjugated second antibody and rhodamine-α-bungarotoxin (50 ng/ml) for 1 hr, washed in PBS again, and mounted in glycerol-*para*-phenylenediamine. To reduce background staining polyclonal antisera to integrins were sometimes incubated overnight at 4°C with adult muscle homogenate in PBS-BSA; the suspension was centrifuged, and the supernatant used for staining. To counterstain basal lamina, antibodies to laminin were included in the first incubation and an appropriate rhodamine-conjugated anti-immunoglobulin antibody was included in the second incubation. In a few cases, sections were fixed in 100% methanol at −20°C for 5–10 min and then treated with urea–glycine as described by Miner and Sanes (1994) before antibodies were applied. This treatment denatured acetylcholine receptors, necessitating the use of the lectin VVA-B_4 (Scott *et al.*, 1988) or antibodies to laminin β2 (Sanes *et al.*, 1990) instead of α-bungarotoxin to mark synaptic sites. Following staining, sections were observed and photographed with epifluorescent illumination, using filters selective for fluorescein or rhodamine.

Immunoblotting

Integrins were isolated from extracts of L8E63 or C2 muscle cells by EHS laminin affinity chromatography, as described in Gu *et al.* (1994). The bound and eluted protein was subject to immunoblot analysis using a 1:500 dilution of the anti-α7 antisera. To block antibody reactivity, undiluted sera were incubated for 30 min with an equal volume of PBS containing 200 μg/ml of the respective peptide, then diluted to 1:500, and used immediately.

To detect integrins in adult muscle, rat diaphragms were minced, frozen in liquid nitrogen, then extracted overnight at 4°C in 1% Triton X-100, 1% sodium deoxycholate, 0.05% SDS, 10 m*M* EDTA, 20 m*M* Tris, pH 8.0, 150 m*M* NaCl, and 1 m*M* phenylmethylsulfonyl fluoride. The suspension was centrifuged at 13,000*g* for 3 min, and the solubilized material was then denatured by boiling in 2% SDS, separated on an SDS–polyacrylamide gel, and blotted as above.

RESULTS

Distribution of Integrin Chains in Adult Muscle

Figure 1a shows a cryostat section of human adult skeletal muscle stained with a monoclonal antibody specific for the integrin β1 subunit. All cells that abut basal laminae were β1-positive, including extrafusal (ordinary) muscle fibers, intrafusal muscle fibers of muscle spindles, capsular cells that enwrap intrafusal fibers, smooth muscle and endothelial cells of intramuscular arteries, endothelial cells of capillaries, Schwann cells that enwrap nerve fibers, and perineurial cells that enwrap fascicles of axon-Schwann cell units. Moreover, both synaptic and extrasynaptic portions of the muscle fiber surface were β1-positive, as shown by double-staining of sections with rhodamine-α-bungarotoxin to mark synaptic sites (Figs. 1b and 1b′). The level of β1-immunoreactivity was consistently higher in synaptic than in extrasynaptic areas, as noted previously in chick muscle by Bozyczko *et al.* (1989). The synaptic enrichment could reflect increased density of β1 in synaptic membranes, increased postsynaptic surface area due to junctional folds, and/or the presence of closely apposed pre- and postsynaptic membranes (Sanes and Chiu, 1983).

To determine which integrins are present at synaptic sites, we used a panel of antibodies specific for the 10 α subunits known to associate with the β1 subunit: α1–α9 and αv. Sections of human, rat, and mouse muscle were stained as appropriate, based on the known species specificities of the antibodies. Results of this survey are summarized in Table 1, and examples are shown in Figs. 1c–1l. All of the antibodies were active, as demonstrated by specific staining of intramuscular structures or nonmuscle control tissues (Table 1). However, antibodies to only two subunits, α1 and α7, stained synaptic sites intensely (Figs. 1h and 1l, respectively). For α1, no staining of extrasynaptic membrane was detectable; for α7, extrasynaptic membrane was clearly stained, but less intensely so than synaptic sites. In each case, the specificity of the staining was confirmed by use of two independently derived monoclonal antibodies (TS2/7 and 3A3 for α1; H36 and O26 for α7; see Materials and Methods). Thus, two integrin subunits, α1 and α7, are concentrated at the neuromuscular junction.

Integrin α1 Is Expressed by Presynaptic Cells

The synaptic localization of integrin α1 could reflect its concentration in pre- and/or postsynaptic membranes. To distinguish between these possibilities, we stained rat muscle with a well-characterized anti-α1 monoclonal antibody, 3A3 (Tawil *et al.*, 1990). At high magnification, it was apparent that α1 was present on processes of the Schwann cells that cap nerve terminals (Figs. 2c and 2d). This localization was consistent with that observed in human muscle, in which α1-immunoreactivity appeared to be concentrated external to the bungarotoxin-stained postsynaptic membrane (Figs. 1c and 1d). As an additional test, we stained sections of muscle that had been denervated 5 or 7 days previously. Denervation leads to degeneration of the nerve terminal and rearrangement of Schwann cell processes, but does not directly disrupt the postsynaptic apparatus. Efficacy of denervation was verified by an absence of staining

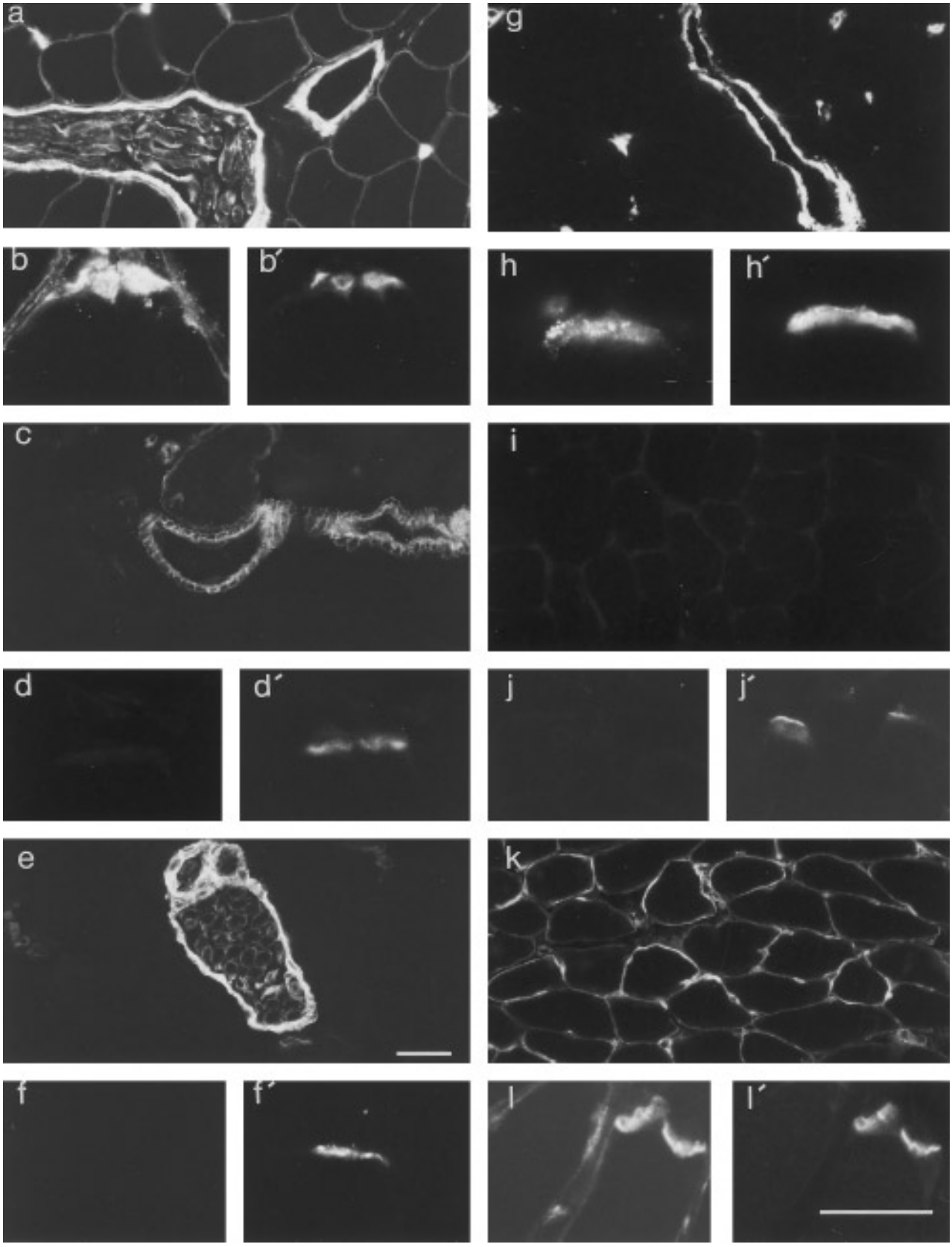
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TABLE 1
Distribution of Integrin Subunits in Adult Muscle

	Muscle fibers		Nerve trunks		Blood vessels	
	Synaptic	Extrasynaptic	Perineurium	Endoneurium	Arterioles	Capillaries
α1	+[a]	−	+	−	+	+
α2	−	−	−	+[b]	+	−
α3	±	−	+	−	+	−
α4	−	−	−	−	−	−
α5	−	−	+	−	+	−
α6	−	−	+	+	+	+
α7	+	+	+	−	+	±
α8	−	−	−	−	+	−
α9[c]	±	±	−	−	−	−
αv	±	±	+	+	+	+
β1	+	+	+	+	+	+

Note. Cryostat sections of human (α1–6, α8, α9, αv, β1), mouse (α4), and rat (α7–9) muscles were stained with subunit-specific monoclonal antibodies to integrins. Sources of antibodies are listed under Materials and Methods. (+) Strong staining; (±) weak or variable staining; (−) no detectable staining. Staining of arterioles was heterogeneous (endothelium, smooth muscle, or both); a + in this column does not distinguish among patterns.

[a] Staining was presynaptic; muscle fiber was unstained.

[b] Stained only a subset of endoneuria.

[c] Although skeletal muscle fibers were barely stained by anti-α9, this antibody stained the esophagus intensely, confirming results of Palmer *et al.* (1993).

for the synaptic vesicle protein, SV2 (data not shown). Unexpectedly, α1 levels increased in a subset of interstitial cells near the damaged nerve (Figs. 2a and 2b). These cells may be Schwann cells, but might also correspond to perisynaptic fibroblast-like cells that are known to proliferate following nerve section (Gatchalian *et al.*, 1989; Weis *et al.*, 1991). In contrast to this interstitial increase, however, levels of synaptic α1-immunoreactivity were dramatically reduced by 5 days after denervation, and no α1 immunoreactivity appeared on muscle fibers per se (Fig. 2e).

To learn when α1 appears at the synapse, we stained muscles from postnatal rats. Staining for α1 was barely visible at Postnatal Day (P) 0 (data not shown), but was clearly detectable and selectively associated with synaptic sites by P4 (Fig. 2f).

Characterization of Antisera Specific for the Integrin α7A, B, and C Isoforms

Three classes of integrin α7 cDNAs have been isolated (Song *et al.*, 1993; Ziober *et al.*, 1993; Collo *et al.*, 1993). All three share sequences that encode predicted extracellular and transmembrane domains, but they encode distinct intracellular domains of 58 (α7A), 77 (α7B), and 18 (α7C) amino acids (Fig. 3a). Sequence analysis of the α7 gene suggests that alternative splicing leads to generation of 3 mRNAs (Fig. 3b). Interestingly, the variation in exon structure leads to use of different reading frames in the three isoforms. As one consequence, the α7A mRNA is longer than that of α7B, but the latter encodes a longer protein. Similarly, a 3′ exon that encodes untranslated sequence in α7A and α7B encodes the carboxyl terminus of the α7C protein (Song *et al.*, 1993). RNAs encoding all three splice forms are present in muscle cell lines, and all three share extracellular sequences recognized by the monoclonal antibodies that we used. Therefore, it was possible that any or all of the α7 isoforms were present at synaptic sites. To clarify this issue, we generated antibodies to synthetic peptides derived from cytoplasmic sequences unique to each of the three splice forms. Positions of the peptides are shown in Fig. 3a, and their sequences are given under Materials and Methods.

To show that the antisera recognized α7 proteins, we isolated laminin-binding proteins from cultured muscle cells and probed them on immunoblots (Fig. 3c). Antisera to all

FIG. 1. Distribution of integrin subunits in skeletal muscle. Sections of human (a–h), mouse (i, j), or rat (k, l) muscle were stained for integrin β1 (a, b), α1 (g, h), α3 (c, d), α4 (i, j), α6 (e, f), or α7 (k, l). Neuromuscular junctions were marked by costaining with rhodamine-α-bungarotoxin (b′–l′). Low-power figures show staining of muscle fibers, perineurium, endoneurium, blood vessels, and capillaries (summarized in Table 1). Synaptic sites are positive for the α1, α7, and β1 subunits, but bear no detectable α1, α3, α4, or α6. Some synaptic sites were faintly α3-positive. Bar in e is 25 μm for a, c, e, g, i, k; bar in l′ is 25 μm for b, d, f, h, j, l.

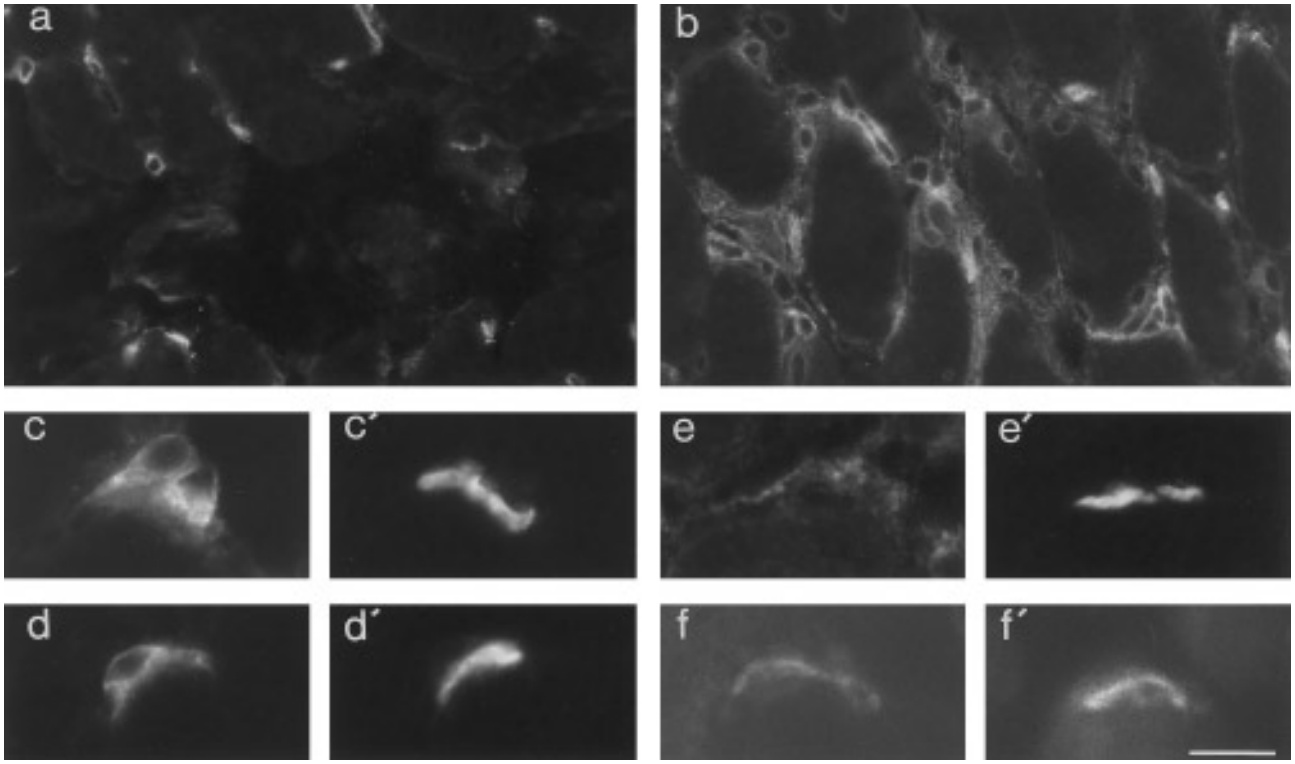

FIG. 2. The integrin α1 subunit is associated with presynaptic elements at the neuromuscular junction. Sections of normal adult (a, c, d), denervated adult (b, e), and neonatal (P4) rat muscle (f) were doubly labeled with anti-integrin α1 (a–f) and rhodamine-α-bungarotoxin (c′–f′). (a) Capillaries are α1-positive, but muscle fibers and interstitial cells are α1-negative in normal adult muscle. (b) In 1-week denervated muscle, anti-α1 stains interstitial cells near the damaged nerve, but muscle fiber surfaces remain α1-negative. This field was chosen to show an unusually dense accumulation of α1-positive cells. (c, d) Higher magnification views of normal neuromuscular junctions show that synapse-associated immunoreactivity is associated with Schwann cell processes. (e) α1 staining is markedly decreased at synaptic sites following denervation. (f) α1 immunoreactivity is present at neuromuscular junctions by P4. Bar is 25 μm for a, b and 10 μm for c–f.

three isoforms recognized proteins of ~120 kDa, which corresponded to the apparent molecular weights of authentic integrins α7A–C. As the isoforms are predicted to differ in mass by <6 kDa, we did not expect that the full-length α7A–C proteins would be distinguishable in our gel system. Following reduction, however, antisera to each isoform recognized distinct smaller bands of 30–40 kDa, which corresponded to known proteolytic products of α7 (Song *et al.*, 1992, 1993). Moreover, the sizes of these fragments were consistent with predicted differences among isoforms: assuming that all isoforms are cleaved at the same site and that their glycosylation is equivalent, α7B is expected to be 2 kDa longer than α7A and 6 kDa longer than α7C. Finally, incubation of anti-α7A and α7B with immunizing peptides abolished their immunoreactivity. Reactivity of anti-α7C on immunoblots was inconsistently inhibited by the short peptide that had been used as immunogen, perhaps reflecting a higher affinity of the antiserum for the native protein than the peptide. However, the ability of this antiserum to recognize laminin-binding proteins of appropriate molecular weights, along with the ability of the immunizing peptide to block immunostaining by the antiserum (see below) provided strong evidence for antibody specificity. In addition, these results indicate that the α7A–C isoforms all bind laminin.

To ask whether α7A–C isoforms were present in adult rat muscle, detergent extracts were fractionated by gel electrophoresis and probed by immunoblotting. Antisera to all three α7 isoforms recognized bands of 120–130 kDa in nonreduced material and bands of 30–40 kDa in reduced material (Fig. 3d and data not shown). Bands of 65–75 kDa were also detected with anti-α7A and anti-α7B in some but not all experiments; these may represent additional proteolytic fragments (Song *et al.*, 1992, 1993). Integrin α7A and α7B RNAs have been detected in muscle by RT–PCR (Song *et al.*, 1993; Ziober *et al.*, 1993; Collo *et al.*, 1993); the present evidence is the first for the expression of α7C in adult muscle.

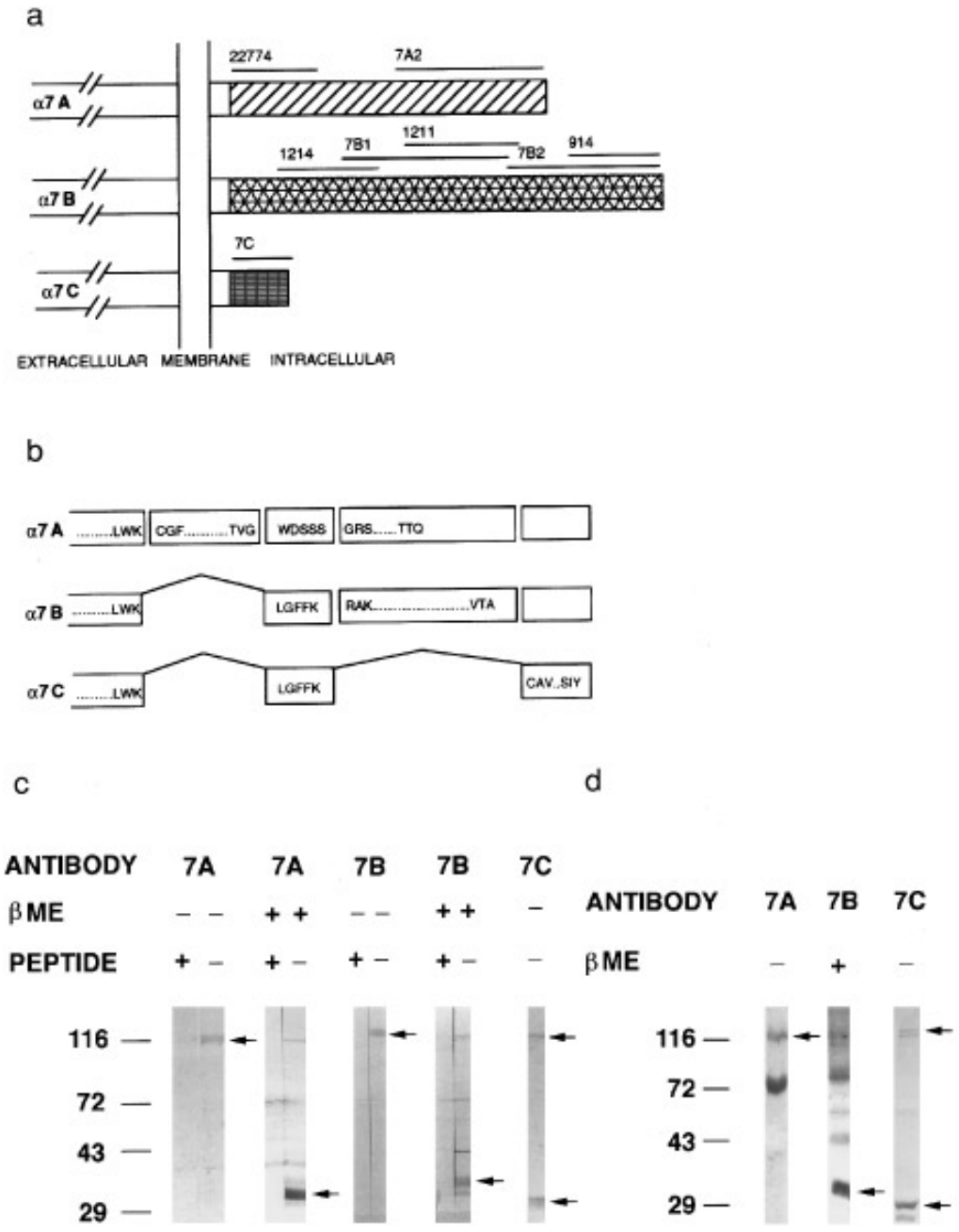

FIG. 3. Structure of the integrin α7A, α7B, and α7C isoforms and generation of antisera specific for each. (a) Sketch of the deduced structure of the A–C isoforms of integrin α7. (b) Sketch of the splicing patterns that have been hypothesized to generate their mRNAs (based on data in Song *et al.*, 1993, and Ziober *et al.*, 1993). As described in the text, the three isoforms have completely divergent sequences beginning six amino acids after the deduced transmembrane segment. Positions of peptides used to generate antisera are marked in a. (c) Laminin-binding proteins were affinity- purified from muscle cells and subjected to immunoblotting using antisera to peptides derived from unique α7A, α7B, or α7C sequences. Unreduced or β-mercaptoethanol (βME)-reduced samples were electrophoresed through SDS–polyacrylamide gels and transferred to nitrocellulose. Blots were incubated with antisera in the presence or absence of their respective immunogenic peptides. Specific bands corresponding to full-length α7 (~120 kDa) and to disulfide-linked proteolytic fragments (30–40 kDa; Song *et al.*, 1992, 1993) are marked by arrows. Other bands persisted in the presence of the immunizing peptide and are therefore considered to represent nonspecific immunoreactivity. In all cases, molecular weights of the specific immunopositive bands were consistent with previous studies and/or predictions from the sequence. Sources of integrins were C2A3 cells (7A βME–), adult mouse muscle (7A βME+), or E63 cells (7B βME–, 7B βME+, and 7C). (d) Detergent extracts of adult rat muscle were probed with the same antisera used in c. Major reactive bands from adult muscle are similar in mobility to those in the laminin-binding fraction isolated from cultured muscle cells. Bands of 65–75 kDa may correspond to proteolytic fragments described by Song *et al.* (1992, 1993).

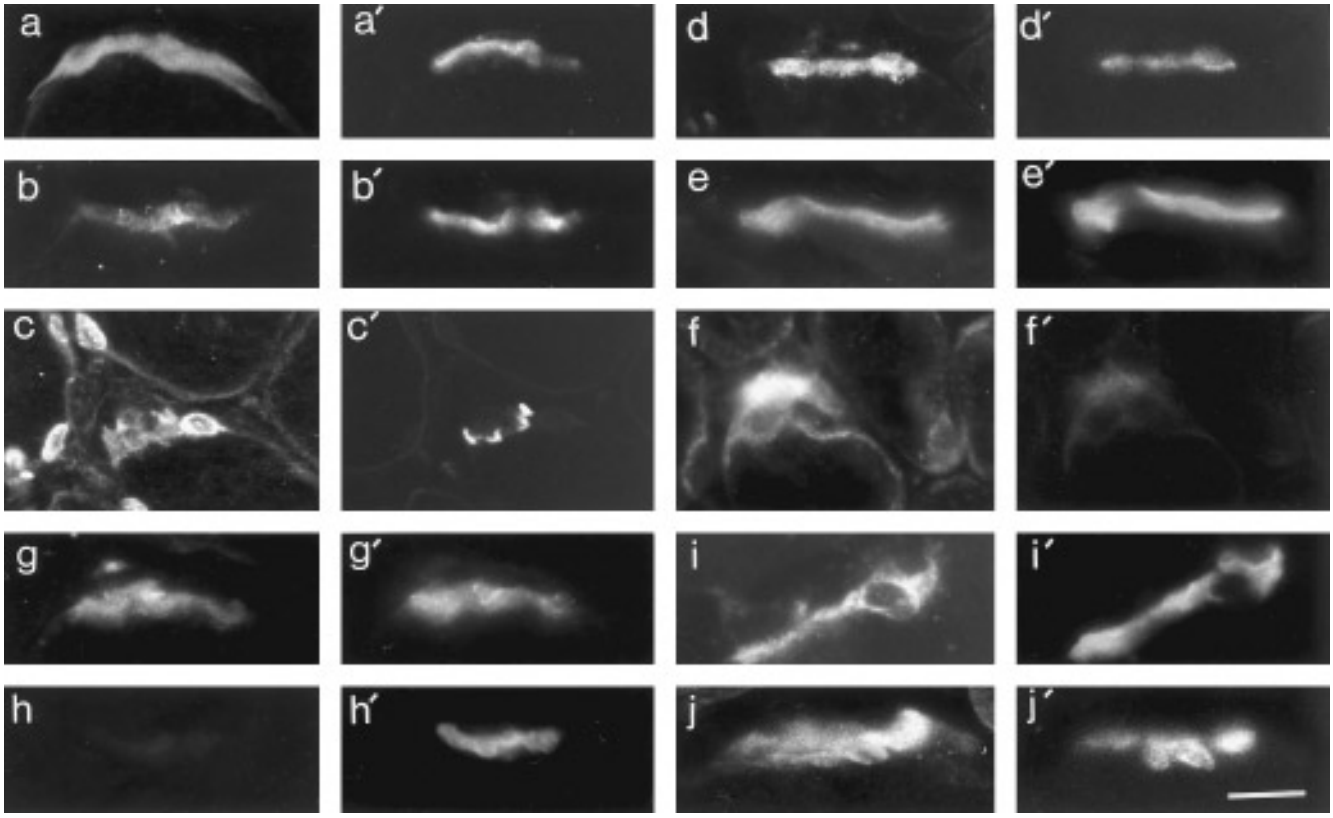

FIG. 4. Differential distribution of α7A–C isoforms at the neuromuscular junction. Antibodies specific for α7A (a, d, i), α7B (b, e, g, h, j), and α7C (c, f) were used to stain adult human (a–c), rat (d–h), or denervated rat (i, j) muscles. Neuromuscular junctions were visualized by costaining sections with rhodamine-α-bungarotoxin (a′, b′, d′, e′, g′–j′) or anti-laminin β2 (c′, f′). Sections stained for α7C were pretreated with urea/glycine (Miner and Sanes, 1994). (a–f) α7A and B are concentrated at synaptic sites in both human and rat muscle, whereas α7C is present both synaptically and extrasynaptically. Intensely stained circular profiles in c may be capillaries, but this staining was seen inconsistently and was not seen in rats (f). (g, h) Staining by anti-α7B was abolished by preincubation with the immunizing peptide (h), but not with an irrelevant peptide (g). Similar results were obtained with anti-α7A and C. (i, j) Synaptic accumulations of α7A (i) and α7B (j) persist following denervation, demonstrating that it was associated with the postsynaptic membrane. Bar is 25 μm for c and f and 10 μm for all other parts.

Integrins α7A and α7B Are Synapse-Specific, but α7C Is Not

Sections of adult human, rat, and mouse muscle were stained with antibodies specific for each α7 integrin isoform. Similar results were obtained in all three species and, for α7B, with antisera generated to four independent peptides: α7A and α7B were concentrated at synaptic sites and undetectable extrasynaptically, whereas α7C was present at similar levels in synaptic and extrasynaptic portions of the muscle fiber surface (Figs. 4a–4f). Staining with antisera to all three alternate cytoplasmic domains was blocked by preincubation with the peptide that had been used as immunogen (see Fig. 4h, for example) but not by preincubation with a heterologous peptide (see Fig. 4g, for example). Denaturation of sections with methanol or urea/glycine neither abolished synaptic immunoreactivity nor revealed extrasynaptic immunoreactivity for α7A and α7B, suggesting that masking of epitopes in extrasynaptic regions (Song *et al.*, 1993) did not account for the synapse-specific staining (data not shown). In contrast, denaturation of sections in urea/glycine was required to visualize α7C, possibly because the hydrophobic peptide used as immunogen was inaccessible in the native protein. Synaptic staining coincided with accumulations of acetylcholine receptors and persisted in denervated muscle (Figs. 4i and 4j), indicating that α7 integrins were associated with the postsynaptic membrane. These results demonstrate that α7A–C integrins are associated with the postsynaptic membrane at the neuromuscular junction, with α7A and α7B being exclusively synaptic.

α7B Integrin Is Present in Peripheral Nerve and Blood Vessels but α7A and α7C Are Not

Antibodies that recognized extracellular epitopes common to all of the α7 isoforms stained not only skeletal

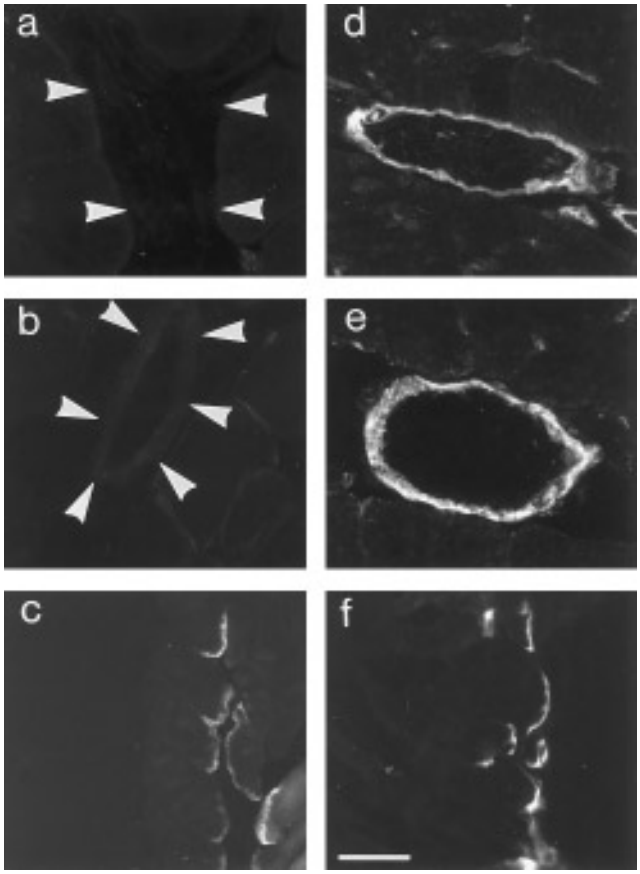

FIG. 5. Differential distribution of α7A and α7B in nonsynaptic intramuscular structures. Section of rat (a, b, d, e) or mouse (c, f) muscle were stained with antisera to integrin α7A (a–c) or 7B (d–f). Integrin α7B but not α7A is present in the perineurium of intramuscular nerves (a, d), and in the walls of intramuscular arterioles (b, e). Positions of a nerve trunk and an anteriole are indicated by arrowheads in a and b, respectively. Both are present at myotendinous junctions (c, f), and neither is present in endoneurium (a, d). Bar, 25 μm.

muscle fiber surfaces, but also perineurial cells in peripheral nerves and smooth muscle cells in arterioles (Table 1). We used the isoform-specific antisera to determine which isoforms were present in these cells. As shown in Figs. 5d and 5e, anti-α7B stained perineurium and arterioles; anti-α7A (Figs. 5a and 5b) and anti-α7C (not shown) did not. In contrast, antibodies to α7A and α7B but not α7C stained myotendinous junctions (Figs. 5c and 5f and data not shown), which had previously been shown to be intensely α7-positive in birds (Bao *et al.*, 1993). Importantly, all structures stained by the pan-α7 antibodies were stained by at least one of the isoform-specific antisera. Together, these patterns (summarized in Table 2 for mouse; similar results were obtained in rat and human muscle) demonstrate that the staining seen with antibodies to a domain shared by all three splice forms represents the sum of distinct expression patterns for each isoform. In addition, our results confirm at the protein level a conclusion reached from RNA analysis (Collo *et al.*, 1993), that α7A expression is muscle-specific, whereas α7B is expressed in some nonmuscle cells.

α7A–C Integrin Expression Patterns Are Distinct during Development

We used rat muscle to learn when α7 isoforms become localized to synaptic sites. At P0, α7A was undetectable, either synaptically or extrasynaptically (Fig. 6a). Low levels of α7A immunoreactivity were detectable at synaptic sites by P4 (not shown), and clear synaptic accumulations of this isoform were present at P7 (Fig. 6d). In contrast, α7B, which was synapse-specific in the adult, was present both synaptically and extrasynaptically at birth, as well as in intracellular, presumably membranous, structures (Fig. 6b). Concentration of α7B at synaptic sites was evident by P7 (Fig. 6e), and this isoform had become exclusively synaptic by P10 (not shown). Finally, α7C, was present throughout the muscle by P0, but was predominantly intracellular (Fig. 6c). At later stages, intracellular staining was lost but surface-associated staining was not (Fig. 6f). Thus, each α7 isoform is regulated in a distinct way, and the α7A and B isoforms, which are both concentrated at synapses in adults, become synapse-specific by different developmental mechanisms.

TABLE 2

Expression of α7 Isoforms in Normal and β2 Laminin-Deficient Mice

		Integrin chain			Laminin chain	
Cell type	Phenotype	α7A	α7B	α7C	β2	β1
Muscle fiber						
Synaptic	+/+, +/−	+	+	+	+	−
	−/−	+	−	+	−	+
Extrasynaptic	+/+, +/−	−	−	+	−	+
	−/−	−	−	+	−	+
Myotendinous	+/+, +/−	+	+	−	+	−
junction	−/−	↓	↓	−	−	−
Peripheral nerve						
Perineurium	+/+, +/−	−	+	−	+	−
	−/−	−	−	−	−	+
Endoneurium	+/+, +/−	−	−	−	−	+
	−/−	−	−	−	−	+
Blood vessels						
Arterioles	+/+, +/−	−	+	−	+	−
	−/−	−	↓	−	−	−
Capillaries	+/+, +/−	−	−	?	−	+
	−/−	−	−	?	−	+

Note. (+) Strong staining; (−) no detectable staining; (↓) present but lower than in controls; (?) inconsistent staining among samples.

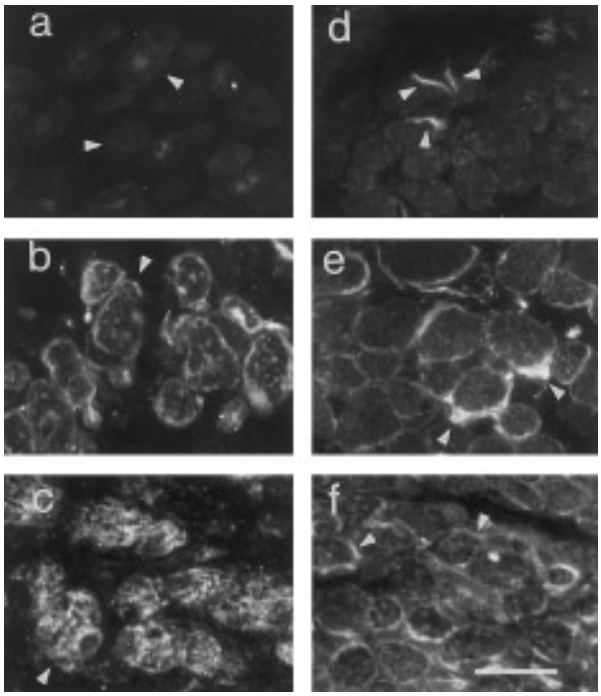

FIG. 6. Differential regulation of α7A–C in developing muscle. Sections from P0 (a–c) or P7 (d–f) rats were stained with antibodies specific for integrin α7A (a, d), α7B (b, e), or α7C (c, f). Positions of synaptic sites, determined by double staining with rhodamine-α-bungarotoxin or anti-laminin β2, are marked by arrows. α7A appears at synaptic sites postnatally, α7B is lost from extrasynaptic areas postnatally, and α7C is present both synaptically and extrasynaptically in neonates and adults. Bar, 25 μm.

α7B Integrin Is Absent from Neuromuscular Junctions of Laminin β2- Deficient Mice

The intramuscular distribution of α7B integrin documented above parallels the distribution of laminin β2 reported previously (Sanes and Chiu, 1983; Sanes *et al.*, 1990): both are concentrated at synaptic sites and myotendinous junctions in muscle, but are absent from extrasynaptic portions of the muscle fiber surface; both are present in perineurium but not endoneurium of peripheral nerve branches; and both are present in arterial vessels but not capillaries. In light of these parallels, it was of interest to assess the distribution of the α7 integrin isoforms in muscles of laminin β2-null mutant mice that we recently generated and characterized. Neuromuscular junctions form in these mice, but both pre- and postsynaptic structures develop aberrantly, and most mutant homozygotes die in the third or fourth postnatal week (Noakes *et al.*, 1995a).

Immunostaining with antisera specific for α7B integrin revealed that the distribution of this isoform was markedly but selectively affected by laminin β2 deficiency: α7B was absent from the neuromuscular junctions and perineurium of laminin β2 mutant mice at P19, but was present, albeit at reduced levels, at myotendinous junctions and blood vessels (Fig. 7b and Table 2). These results suggest that a potential ligand of α7 integrins (see Discussion) can affect the distribution of its receptor. In contrast, the intramuscular distribution of integrin α7A and α7C were qualitatively similar in β2 mutant homozygotes and littermate controls: α7A was concentrated at synaptic sites and myotendinous junctions, α7C was present throughout the muscle fiber surface, and neither isoform was detectable in nerves or blood vessels (Fig. 7a and Table 2). However, levels of α7A at myotendinous junctions were markedly lower in mutants than in normal mice. The loss of integrin α7B but not α7A from mutant synapses suggests that the distribution of α7 integrin isoforms is differentially influenced by the extracellular matrix.

Finally, we investigated one potential explanation for the selective loss of integrin α7B from only a subset of structures that are normally laminin β2- and integrin α7B-positive—i.e., synaptic sites and perineurium but not blood vessels and myotendinous junctions. In normal muscles, the cellular surfaces that are integrin α7B-negative (extrasynaptic portions of the muscle fiber, endoneurium, and capillaries) bear basal laminae rich in the laminin β1 chain (Table 2). Recently, in studying renal defects in the laminin β2 mutant mouse, we found that the glomerular basement membrane, which is normally β2-positive, compensates for the loss of β2 by maintaining or accumulating β1 (Noakes

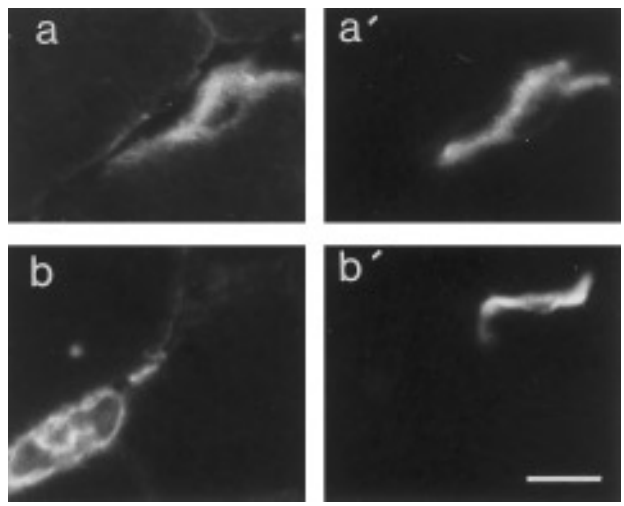

FIG. 7. Absence of α7B integrin from neuromuscular junctions of laminin β2-deficient mice. Sections from P19 mutant homozygotes (Noakes *et al.*, 1995) were stained with anti-α7A(a) or anti-α7B(b), plus rhodamine-α-bungarotoxin (a′, b′). Synaptic sites in normal mice are rich in both α7A and α7B (not shown). α7B is absent from mutant synaptic sites, but present in nearby blood vessels. Bar, 10 μm.

et al., 1995b). In contrast, loss of $\beta 2$ from intrarenal arterioles is not accompanied by accumulation of $\beta 1$ (J. Miner and J.R.S., unpublished results). Accordingly, we stained wild-type and laminin $\beta 2$ mutant mice with a monoclonal antibody specific for laminin $\beta 1$ (Abrahamson *et al.,* 1989). As summarized in Table 2, the two $\beta 2$-positive sites that lost integrin $\alpha 7$B in the mutant (neuromuscular junctions and perineurium) also acquired laminin $\beta 1$, whereas the two $\beta 2$-positive sites that retained integrin $\alpha 7$B in the mutant (blood vessels and myotendinous junctions) did not acquire laminin $\beta 1$. Thus, concentration of integrin $\alpha 7$B may be promoted by laminin $\beta 2$ and/or inhibited by laminin $\beta 1$ in the overlying basal lamina.

DISCUSSION

The $\beta 1$ integrins were discovered as antigens that mediated adhesion of muscle cells to the extracellular matrix (Neff *et al.,* 1982; Greve and Gottlieb, 1982). Subsequently, they have been implicated in several steps in myogenesis, including the migration, differentiation, and fusion of myoblasts (Jaffredo *et al.,* 1987; Menko and Boettinger, 1987; Rosen *et al.,* 1992). The patterns of expression described here suggest that integrins are also involved in the maturation or maintenance of the neuromuscular junction: (1) Integrin $\alpha 1$ is concentrated on presynaptic structures at the adult neuromuscular junction. (2) Integrin $\alpha 7$ is concentrated in the postsynaptic membrane. (3) The $\alpha 7$A–C proteins, generated by alternative splicing, are differentially distributed at developing and adult neuromuscular junctions. (4) Removal of laminin $\beta 2$ from the synaptic basal lamina leads to loss of integrin $\alpha 7$B but not $\alpha 7$A from the underlying postsynaptic membrane.

Synaptic Integrins

We assessed the distribution of all 10 of the integrin α subunits that have been shown to form dimers with $\beta 1$. In fact, our panel of antibodies should have detected all integrins so far described in nonlymphoid tissues: other known β subunits dimerize with 1 or more of these 10 α subunits ($\beta 3$,5,6, and 8 with αv, $\beta 4$ with $\alpha 6$, and $\beta 7$ with $\alpha 4$; Sonnenberg, 1993). The two subunits that we found to be concentrated at the neuromuscular junction, $\alpha 1$ and $\alpha 7$, have so far been found only in association with $\beta 1$. We therefore suspect, but have not proven, that the synapse-specific integrins are $\alpha 1\beta 1$, $\alpha 7$A$\beta 1$, and $\alpha 7$B$\beta 1$.

Although integrin $\alpha 1$ is present on developing muscle cells (Duband *et al.,* 1992), it appears to be confined to presynaptic structures at the adult neuromuscular junction. At least part of the synaptic immunoreactivity is associated with the Schwann cells that cap nerve terminals, whereas preterminal Schwann cells, which myelinate motor axons, are not detectably $\alpha 1$-positive. This distinction is consistent with recent studies *in vitro,* which have shown that integrin $\alpha 1\beta 1$ is abundant on isolated and nonmyelinating Schwann cells, but is down-regulated during myelination (Einheber *et al.,* 1993; Fernandez-Valle *et al.,* 1994; Mechtersheimer *et al.,* 1994). It is possible that $\alpha 1$-immunoreactivity is also present on the membrane of the motor nerve terminal, as $\alpha 1$ is expressed by several neuronal types *in vitro* (Tomaselli *et al.,* 1993), and levels of $\alpha 1$-immunoreactivity decrease markedly following denervation, at a time when nerve terminals have degenerated but Schwann cells persist (Fig. 2). Given the intense immunoreactivity of the Schwann cell, electron microscopic methods will be required to determine whether the closely apposed axonal membranes are also $\alpha 1$-positive.

The $\alpha 7\beta 1$ integrin was identified independently as the predominant laminin-binding integrin of myoblasts (von der Mark *et al.,* 1991), a laminin-binding integrin of melanoma cells (Kramer *et al.,* 1991), and a cell surface antigen on cultured muscle cells (Kaufman *et al.,* 1985; Song *et al.,* 1992). Subsequent studies on rats and chicks have shown that $\alpha 7\beta 1$ is differentially expressed by developing primary and secondary myotubes, that its levels decline as muscle fibers mature but remain detectable in adulthood, and that it is concentrated at myotendinous junctions (Song *et al.,* 1992; George-Weinstein *et al.,* 1993; Bao *et al.,* 1993). We show here that $\alpha 7$ levels remain high at the neuromuscular junction after they decline extrasynaptically. The correspondence of $\alpha 7$-immunoreactivity with acetylcholine receptors (marked with α-bungarotoxin) and the persistence of immunoreactivity following denervation demonstrate that most if not all of the $\alpha 7$ at synaptic sites is associated with the postsynaptic membrane. Moreover, use of isoform-specific antibodies show that $\alpha 7$A and $\alpha 7$B are present synaptically but undetectable extrasynaptically, with the extrasynaptic immunoreactivity accounted for by $\alpha 7$C. To our knowledge, $\alpha 7$A and $\alpha 7$B are the first integrin subunits shown to be specifically associated with synaptic membranes anywhere in the central or peripheral nervous system. It will be of interesting to ask whether other integrin subunits are concentrated at other synapses.

In elucidating the roles that synaptic integrins play, it will be important to identify their ligands in the synaptic cleft. Both $\alpha 1\beta 1$ and $\alpha 7\beta 1$ are known to bind to laminin-1 (chain composition $\alpha 1/\beta 1/\gamma 1$) and $\alpha 1\beta 1$ also binds to tumor derived collagen IV [chain composition $(\alpha 1)_2(\alpha 2)_1$] (Belkin *et al.,* 1990; Colognato-Pyke *et al.,* 1995; Gu *et al.,* 1994; Kramer *et al.,* 1991; Lein *et al.,* 1991; Tawil *et al.,* 1990; von der Mark *et al.,* 1991). Synaptic basal lamina contains distinct forms of both laminin and collagen IV: a novel laminin α chain in place of $\alpha 1$, laminin $\beta 2$ in place of $\beta 1$, and collagens $\alpha 3$–5(IV) in place of $\alpha 1$,2(IV) (Sanes *et al.,* 1990; Green *et al.,* 1992; Miner and Sanes, 1994; J. Miner and J.R.S., unpublished). We speculate that integrins $\alpha 1\beta 1$, $\alpha 7$A$\beta 1$, and $\alpha 7$B$\beta 1$ may bind with particularly high affinity and/or respond in distinct ways to synaptic laminins or collagens IV.

Alternative Splicing

The α7A–C isoforms are predicted to bear distinct cytoplasmic domains, beginning six residues after the putative transmembrane domain (Collo *et al.*, 1993; Ziober *et al.*, 1993; Song *et al.*, 1993). By using isoform-specific antisera, we have confirmed at the protein level two conclusions previously reached from RNA analysis: that α7A is muscle-specific while α7B is more widely distributed and that expression of α7B precedes that of α7A during myogenesis (Collo *et al.*, 1993; Ziober *et al.*, 1993; Song *et al.*, 1993). In addition, we have shown that the three isoforms are localized differently within muscle fibers: (1) α7A and α7B are concentrated at adult neuromuscular and myotendinous junctions, but α7C is not. (2) α7A is synapse-specific from its first appearance, whereas α7B is initially present in extrasynaptic membrane as well. (3) α7B is absent from synapses of laminin β2-deficient mice, whereas α7A and α7C are unperturbed. These results establish that alternatively spliced variants of a single integrin subunit can be differentially regulated and localized within a single cell.

Alternative splicing has been shown to lead to generation of variants of several integrin α and β chains (Sonnenberg, 1993). The C form, which has an extremely short cytoplasmic domain, is so far unique to the α7 chain (Song *et al.*, 1993), but the A and B forms are homologous to alternatively spliced forms of the α3 and α6 integrin subunits (Cooper *et al.*, 1991; Hogervorst *et al.*, 1991; Tamura *et al.*, 1991; Sonnenberg, 1993). This conservation suggests that the alternative cytoplasmic domains play distinct and important roles. To date, however, documented differences between the ligand-binding and activation properties of the α6A and α6B isoforms are not impressive (Shaw *et al.*, 1993; Delwel *et al.*, 1993, 1994; Hogervorst *et al.*, 1993). An additional possibility, suggested by our results, is that different cytoplasmic domains may contribute to the differential localization of the variants. How the alternate cytoplasmic domains would lead to differences in distribution remains to be determined. Possibilities include the following: (1) α7A–C integrins might associate with distinct intracellular elements. For example, the cytoplasmic tails of α7A and α7B might be better able than the short α7C tail to interact with integrin-associated cytoskeletal proteins that are concentrated at the neuromuscular and myotendinous junctions (Hall and Sanes, 1993). (2) Synaptic nuclei might be specialized to splice α7A and α7B mRNAs from the α7 transcript, just as they are known to selectively transcribe genes for ''synapse-specific'' proteins (Merlie and Sanes, 1985; Moscoso *et al.*, 1995a). (3) Translational efficiency of the α7A-C mRNAs, or stabilities of the α7A-C proteins may vary among compartments or during development. (4) The α7A–C forms might associate with distinct β chains or β1 variants, or they might covary with alternatively spliced variants in the extracellular domain (α7X1 and α7X2 forms; Ziober *et al.*, 1993; note that none of the antibodies we used distinguish between X1 and X2). In these cases, signals for localization might lie in the extracellular domain of α7 or in the β subunit.

The absence of integrin α7B from laminin β2-deficient endplates not only provides an additional distinction between integrin variants but also provides genetic evidence that the composition of the basal lamina can effect the differentiation of the underlying membrane. Numerous recent studies have documented the importance of the extracellular matrix as a regulator of cellular development and differentiation (reviewed in Adams and Watt, 1993). Indeed, the neuromuscular junction provides a dramatic example: components of synaptic basal lamina can organize differentiation of both the nerve terminal and the postsynaptic membrane (Sanes *et al.*, 1978; reviewed in Sanes, 1995). Few of the receptors that mediate the effects of the synaptic basal lamina have been identified, but the present work suggests that the integrins are involved. In many cases, extracellular ligands exert fine regulatory control by affecting the levels of the receptors through which they signal. The perturbation of integrin α7B distribution in the laminin β2 mutant suggests that laminins may exert such dual effects on integrins.

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